Euphytica **132:** 87–94, 2003.

Allelic structures of UDP-glucose:limonoid glucosyltransferase affect limonoid bitterness in *Citrus unshiu* and *C. sinensis*

Masayuki Kita[1,2,*], Tomoko Endo[1], Takehiko Shimada[1], Takaya Moriguchi[1,2], Yutaka Hirata[3], Shin Hasegawa[4] & Mitsuo Omura[1]

[1]*Department of Citriculture, National Institute of Fruit Tree Science, Shimizuokitsu, Shizuoka 424-0292, Japan;* [2]*Department of Plant, Cell and Environment, National Institute of Fruit Tree Science, Fujimoto, Tsukuba, Ibaraki 305-8605, Japan;* [3]*United Graduate School, Tokyo University of Agricultural and Technology, Fuchu, Tokyo 183-0054, Japan;* [4]*Agricultural Research Service, Western Regional Research Center, USDA, 800 Buchanan Street, Albany, CA94710, USA; (*Author for correspondence: E-mail: mkita@affrc.go.jp)*

Received 13 August 2002; accepted 28 February 2003

Key words: allele, *Citrus*, genetic marker, glucosyltransferase, limonoid

Summary

To develop a molecular indicator for determining the level of limonoid bitterness in Satsuma mandarin (*Citrus unshiu*) and navel orange (*C. sinensis*), the genetic background of the transcription of the limonoid glucosyltransferase gene was characterized in relation to the accumulation of non-bitter limonoid glucosides. Two types of cDNA clones (*CitLGT-1* and *CitLGT-2*) encoding limonoid glucosyltransferase were isolated and characterized. The relationships between the gene expression patterns of *CitLGT*s and the non-bitter limonoid glucoside accumulation as well as the allelic structures of *CitLGT*s were determined. Southern blot analysis and segregation analysis of the progenies between Satsuma mandarin and tangor (*C. unshiu* × *C. sinensis*) indicated that *CitLGT-1* and *CitLGT-2* are transcribed from the alleles in a single locus. The genotype of the *CitLGT* locus in navel orange is homozygous for *CitLGT-1* (*CitLGT-1*/*CitLGT-1*), whereas that in Satsuma mandarin is heterozygous for *CitLGT-1* and *CitLGT-2* (*CitLGT-1*/*CitLGT-2*). The levels of non-bitter limonoid glucosides in navel orange fruit were quite low at the early- to mid-developmental stage due to a defective *CitLGT-2*, whereas both non-bitter limonoid glucoside content and *CitLGT-2* expression in Satsuma mandarin fruit were high throughout fruit development. These results indicate that the expression of *CitLGT-2* is a prerequisite for the accumulation of non-bitter limonoid glucosides at the early- to mid-developmental stage of fruit. The allelism of the two *CitLGT*s, that is, whether the *CitLGT-2* is present or not, is a useful molecular indicator for predetermining the levels of accumulation of non-bitter limonoid glucosides at the early- to mid-developmental stages of Satsuma mandarin and navel orange fruits.

Introduction

Limonoids, the bitter constituents of orange juice, are a group of highly oxygenated triterpenoids observed in the Rutaceae, Meliaceae, and related plant families. Limonin is a major component of limonoids in citrus and lowers the market value of fruit juice. Limonoid bitterness occurs gradually after the juice processing of navel oranges (*Citrus sinensis* Osb.) harvested at the early- to mid-developmental stage of fruit. This bitterness has been referred to as 'delayed bitterness' (Mayer & Beverly, 1968). The mechanism for generating limonin is ascribed to the conversion of a non-bitter precursor, limonoate A-ring lactone (LARL), to limonin under the acidic condition in the juice. This reaction is accelerated by the activation of limonoid D-ring lactone hydrolase (Mayer et al., 1969). Furthermore, if orange fruits are frozen or mechanically damaged, the conversion from LARL to limonin will occur in the citrus fruit itself. Contrary to navel orange, Satsuma mandarin (*C. unshiu* Marc.) fruits, the most popular citrus fruit in East Asia, is less bitter, even from the early- to mid-developmental stages (Hash-

inaga et al., 1977), mainly because of the conversion of LARL to the tasteless limonin glucoside (Ozaki et al., 1995). Although the molecular regulation for this conversion is not yet fully understood, the gene encoding UDP-D-glucose:limonoid glucosyltransferase which catalyzes the conversion of LARL to limonin glucoside was recently isolated from Satsuma mandarin (Kita et al., 2000) based on information of the purified enzyme protein (Hasegawa et al., 1997).

While limonoids give a bitter taste to citrus fruit, they also have potential pharmacological functions (Lam et al., 1994). Limonoids have been shown to be strong inducers of glutathion S-transferase, the activity of which is positively correlated with inhibitory action against carcinogenesis (Lam et al., 1989). Citrus limonoids have been shown to inhibit many types of chemically induced neoplasia using animals and cultured mammalian cells (Lam & Hasegawa, 1989). Limonoid glucosides are tasteless and water-soluble, and the inhibitory effect on carcinogenesis is the same as that of bitter limonoids (Miller et al., 1994).

Tangors are important hybrids of mandarins and sweet oranges (*C. sinensis*). They are used in breeding program to integrate favorable characteristics of both traits, including easy-peeling, seedlessness, and good flavor and color. However, in the case of tangors, it is important to prevent limonoid bitterness derived from *C. sinensis*. Moreover, it takes years to obtain fruit-bearing trees for evaluation of the fruit characteristics. When Satsuma mandarin and navel orange are crossed to develop a non-bitter tangor, molecular markers may be used to predetermine the levels of limonoid bitterness in the progenies at the juvenile stage. Here, we report the isolation of two types of *CitLGT*s (*CitLGT-1* and *CitLGT-2*), which were derived from an allelic *CitLGT* locus. Navel orange, which has delayed bitterness, is homozygous for *CitLGT-1*, whereas Satsuma mandarin, which does not have limonoid bitterness, is heterozygous for *CitLGT-1* and *CitLGT-2*. This difference in allele structure contributes to differences in the accumulation pattern of limonoid glucosides in fruits of navel orange and Satsuma mandarin at the early- to mid-developmental stages.

Materials and methods

Plant materials

All samples used in this study were collected from the experimental field of the National Institute of Fruit Tree Science, Shimizuokitsu (Shizuoka, Japan). Fruit of Satsuma mandarin (*C. unshiu* Marc. cv. Miyagawa-wase) and navel orange (*C. sinensis* Osb. cv. Washington navel) were harvested at 30-day intervals starting 60 days after flowering (DAF). The juice sacs/segment epidermis (edible part) was separated from the fruit and immediately frozen in liquid nitrogen, lyophilized, and kept at –80 °C until use. For the genetic analysis of *CitLGT* alleles, BC_1 segregating progenies between Miyagawa-wase and Kiyomi tangor were used in addition to the parental cultivars: Miyagawa-wase, Kiyomi tangor (Miyagawa-wase × Trovita orange), and Trovita orange (bud mutant of Washington navel).

PCR amplification

Total genomic DNA was isolated from leaves of Satsuma mandarin, navel orange, and the F_1 segregating progenies between Miyagawa-wase and Kiyomi tangor according to the method of Dellaporta et al. (1983). Since *CitLGT* has no intron in the genome (Kita et al., 2000), PCR was performed using the total DNA of Satsuma mandarin and navel orange to obtain the coding region of the *CitLGT* gene under the following conditions: sense primer, LGT-GF (5'-ATG GGA ACT GAA TCT CTT GTT CAT-3'), and antisense primer, LGT-GR (5'-TCA ATA CTG TAC ACG TGT CCG TCG-3'). The reaction was performed for 35 cycles (1 min at 94 °C, 1 min at 56 °C, and 2 min at 72 °C). The amplified fragments were digested with restriction enzymes and electrophoresed on 1.5% agarose gel. After confirmation of polymorphism, the amplified fragments were cloned into a pCR2.1 vector with a TA cloning system (Invitrogen, Groningen, Netherlands) and sequenced to detect nucleotide substitutions among the *CitLGT* candidates using a model 373A sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

Expression analysis of two types of limonoid glucosyltransferase using RT-PCR

Total RNA from fruit was isolated according to Ikoma et al. (1996). First-strand cDNA was synthesized with a Ready-To-Go You-Prime First-Strand Beads (Amersham-Pharmacia Biotech, Little Chalfont, UK). Based on the sequence polymorphisms obtained by the amplified genomic fragments (Figure 1), specific antisense primers LGT-A1 (for *CitLGT-1*; 5'-TCA GCG CGT TCT CCT CCA GCG CCA-3') and LGT-A2 (for *CitLGT-2*; 5'-TCA GCG CGT TCT CCT TCA

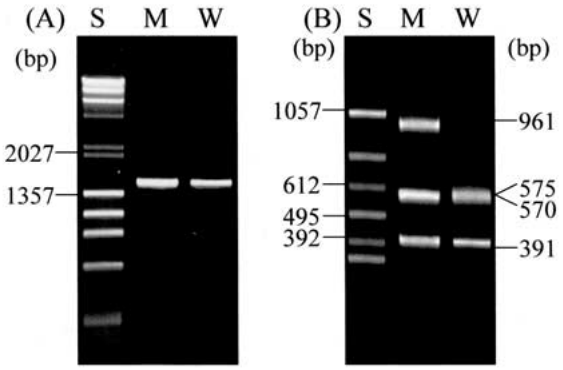

Figure 1. (A) Amplification of cDNA from Miyagawa-wase (M) (*Citrus unshiu*) and Washington navel (W) (*C. sinensis*) using primers LGT-GF and LGT-GR. Size marker(S): λ/*Hin*dIII + $\phi\times$174/*Hae*III. (B) PCR products were digested by *Hinc*II, and separated on 1.5% agarose gel. Size marker (S): $\phi\times$174/*Hinc*II.

GCT CCG-3') were synthesized (Figure 2). PCR was performed with the first-strand cDNA using the common sense primer (LGT-GF) and specific antisense primer (LGT-A1 or LGT-A2) under the conditions described above. To confirm the specificity of the primers, the specific fragmentation pattern of amplification products was checked by electrophoresis after digestion with *Hinc*II or *Mbo*I.

Southern hybridization

For Southern blot analysis, 10 micrograms of total DNA isolated from leaves was digested with *Dra*I, *Eco*RI, and *Xba*I, separated on 1.0% agarose gels, and blotted onto a nylon membrane (Hybond-NX, Amersham-Phrmacia Biotech, Little Chalfont, UK). The blot was hybridized with the probe of the coding region of *CitLGT* from navel orange labeled with Dig-11-dUTP and a Random Primed Labeling kit (Boehringer Mannheim, Germany), and was washed twice with 0.1 × SSC and 0.1% SDS at 65 °C for 15 min and then exposed to X-ray film (RX-U, Fuji Film, Tokyo, Japan).

Measurement of limonin glucoside

Lyophilized fruit samples were extracted by methanol according to the method of Miyake et al. (1992). The extracts were concentrated using a rotary evaporator and dissolved with 0.1 M sodium formate buffer (pH 3.8). In order to eliminate impurities, hesperinase, naringinase, and pectinase were added to this solution and incubated overnight at room temperature. Reaction mixtures were then loaded on a Sep-Pak-C18 column (Waters, Milford, Massachusetts, USA), eluted with methanol, and used for HPLC analysis. HPLC was performed with a Hewlett Packard model 1100 systems using an ODS-Hypersil C18 column (Hewlett Packard, Palo Alto, CA, USA, 125 mm × 4 mm r.d.). Methanol (Phase A) and 10 mM phosphate buffer (Phase B) were used for the mobile phase. The gradient to develop the column was as follows: After an initial 5 min of Phase A:Phase B (10:90), a linear gradient to 51.6% B was developed over 37 min at a flow rate of 1.0 ml/min. The absorbance of the eluent was monitored at 214 nm.

Results and discussion

Using the primers synthesized for amplification of the *CitLGT* coding region (Kita et al., 2000), we successfully amplified fragments of the same size (1,536 bp) by PCR against the genomic DNA of Satsuma mandarin and navel orange (Figure 1A). When the products were digested with *Hinc*II and separated by electrophoresis, polymorphism was observed between the two citrus species (Figure 1B). According to the sequence information, *CitLGT* divides into three fragments (575, 570, and 391 bases) after digestion by *Hinc*II. It was difficult to separate the 575 and 570 bp fragments in agarose gel electrophoresis. Therefore, in the case of navel orange (Washington navel), two bands were observed: one was 391 bp, and the other was overlapping bands of 575 and 570 bp (Figure 1B). On the other hand, in Satsuma mandarin (Miyagawa-wase), an additional band, estimated to be 961 bp, was observed. This result suggests that another type of *CitLGT* also exists in Satsuma man-

```
CitLGT-1      1:ATGGGAACTGAATCTCTTGTTCATGTCTTACTAGTTTCATTCCCCGGCCATGGCCACGTAAACCCGCTCCTGAGGCTCGGCAGACTCCTT 90
CitLGT-2      1:ATGGGAACTGAATCTCTTGTTCATGTCTTACTAGTTTCATTCCCCGGCCATGGCCACGTAAACCCGCTCCTGAGGCTCGGCCGACTCCTT 90
                ---------->                                                                          *
                LGT-GF

CitLGT-1     91:GCTTCAAAGGGTTTCTTTCTCACCTTGACCACACCTGAAAGCTTTGGCAAACAAATGAGAAAAGCGGGTAACTTCACCTACGAGCCTACT 180
CitLGT-2     91:GCTTCAAAGGGTTTCTTTCTCACCTTGACCACACCTGAAAGCTTTGGCAAACAAATGAGAAAAGCGGGTAACTTCACCTACGAGCCTACT 180

CitLGT-1    181:CCAGTTGGCGACGGCTTCATTCGCTTCGAATTCTTCGAGGATGGATGGGACGAAGACGATCCAAGACGCGAAGATCTTGACCAATACATG 270
CitLGT-2    181:CCAGTTGGCGACGGCTTCATTCGCTTCGAATTCTTCGAGGATGGATGGGACGAAGACGATCCAAGACGCGGAGATCTTGACCAATACATG 270
                                                                                      *

CitLGT-1    271:GCTCAACTTGAGCTTATTGGCAAACAAGTGATTCCAAAAATAATCAAGAAAAGCGCTGAAGAATATCGCCCCGTTTCTTGCCTGATCAAT 360
CitLGT-2    271:GCTCAACTTGAGCTTATTGGCAAACAAGTGATTCCAAAAATAATCAAGAAAAGCGCTGATGAATATCGCCCCGTTTCTTGCCTGATCAAT 360
                                                                                 *

CitLGT-1    361:AACCCATTTATCCCTTGGGTCTCTGATGTTGCTGAATCCCTAGGGCTTCCGTCTGCTATGCTTTGGGTTCAATCTTGTGCTTGTTTTGCT 450
CitLGT-2    361:AACCCATTTATCCCTTGGGTCTCTGATGTTGCTGAATCCCTAGGGCTTCCGTCTGCTATGCTTTGGGTTCAATCTTGTGCTTGTTTTGCT 450

CitLGT-1    451:GCTTATTACCATTACTTTCACGGTTTGGTTCCATTTCCTAGTGAAAAAGAACCCGAAATTGATGTTCAGTTGCCGTGCATGCCACTACTG 540
CitLGT-2    451:GCTTATTACCATTACTTTCACGGTTTGGTTCCATTTCCTAGTGAAAAAGAACCCGAAATTGATGTTCAGTTGCCGTGCATGCCACTACTG 540

CitLGT-1    541:AAGCATGATGAAATGCCTAGCTTCTTGCATCCGTCAACTCCTTATCCTTTCTTGAGAAGAGCTATTTTGGGGCAGTACGAAAATCTTGGC 630
CitLGT-2    541:AAGCATGATGAAGTGCCTAGCTTCTTGCATCCGTCAACTCCTTATCCTTTCTTGAGAAGAGCTATTTTGGGGCAGTACGAGAATCTTGGC 630
                    *                                                                          *

CitLGT-1    631:AAGCCGTTTTGCATATTGTTGGACACTTTCTATGAGCTTGAGAAAGAGATTATCGATTACATGGCAAAAATTTGCCCTATTAAACCCGTC 720
CitLGT-2    631:AAGCCGTTTTGCATATTGTTGGACACTTTCTATGAGCTTGAGAAAGAGATTATCGATCACATGGCAAAAATTTGCCCTATTAAACCCGTC 720
                                                                                 *

CitLGT-1    721:GGCCCTCTGTTCAAAAACCCTAAAGCTCCAACCTTAACCGTCCGCGATGACTGCATGAAACCCGATGAATGCATAGACTGGCTCGACAAA 810
CitLGT-2    721:GGCCCTCTGTTCAAAAACCCTAAAGCTCCAACCTTAACCATCCGCGATGACTGCATGAAACCCGATGAATGCATAGACTGGCTCGACAAA 810
                                            *

CitLGT-1    811:AAGCCACCATCATCCGTTGTGTACATCTCTTTCGGCACGGTTGTCTACTTGAAGCAAGAACAAGTTGAAGAAATTGGCTATGCATTGTTG 900
CitLGT-2    811:AAGCCACCATCATCCGTTGTGTACATCTCTTTCGGCACGGTTGTCTACTTGAAGCAAGAACAAGTTGAAGAAATTGGCTATGCATTGTTG 900

CitLGT-1    901:AACTCGGGGATTTCGTTCTTGTGGGTGATGAAGCCGCCGCCTGAAGACTCTGGCGTTAAAATTGTTGACCTGCCAGATGGGTTCTTGGAG 990
CitLGT-2    901:AACTCGGGGATTTCGTTCTTGTGGGTGATGAAGCCGCCGTCTGAAGACTCTGGCGTTAAAATTGTTGGCCTGCCAGATGGGTTCTTGGAG 990
                                            *                                              *

CitLGT-1    991:AAAGTTGGAGATAAGGGCAAAGTTGTGCAATGGAGTCCACAAGAAAAGGTGTTGGCTCACCCTAGTGTTGCTTGCTTTGTGACTCACTGC 1080
CitLGT-2    991:AAAGTTGGAGATAAGGGCAAAGTTGTGCAATGGAGTCCACAAGAAAAGGTGTTGGCTCACCCTAGTGTTGCTTGCTTTGTGACTCACTGC 1080

CitLGT-1   1081:GGCTGGAACTCAACCATGGAGTCGTTGGCATCGGGGGTGCCGGTGATCACCTTCCCGCAATGGGGTGATCAAGTAACTGATGCCATGTAT 1170
CitLGT-2   1081:GGCTGGAACTCAACCATGGAGTCGTTGGCATCGGGGGTGCCGGTGATCACCTTCCCGCAATGGGGTGATCAAGTAACTGATGCCATGTAT 1170

CitLGT-1   1171:TTGTGTGATGTGTTCAAGACCGGTTTAAGATTGTGCCGTGGAGAGGCAGAGAACAGGATAATTTCAAGGGATGAAGTGGAGAAGTGCTTG 1260
CitLGT-2   1171:TTGTGTGATGTGTTCAAGACCGGTTTAAGATTGTGCCGTGGACAGGCAGAGAACAGGATAATTTCAAGGGATGAAGTGGAGAAGTGCTTG 1260
                                            *                                              *
                                            <----------
                                            LGT-A1

CitLGT-1   1261:CTCGAGGCCACGGCCGGACCTAAGGCGGTGGCGCTGGAGGAGAACGCGCTGAAGTGGAAGAAGGAGGCGGAGGAAGCTGTGGCCGATGGT 1350
CitLGT-2   1261:CTCGAGGCCACGGCCGGACCTAAGGCGGCGGAGCTGAAGGAGAACGCGCTGAAGTGGAAGAAGGAGGCGGAGGAAGCTGTGGCCGATGGT 1350
                                        * *  *  <----------
                                               LGT-A2

CitLGT-1   1351:GGCTCGTCGGATAGGAACATTCAGGCTTTCGTTGATGAAGTAAGAAGGACAAGTGTCGAGATTATAACCAGCAGCAAGTCGAAGTCAATC 1440
CitLGT-2   1351:GGCTCGTCGGATAGGAACATTCAGGCTTTCGTTGATGAAGTAAGAAGGAGAAGTGTCGAGATCATAACCAGCAGCAAGTCGAAGTCAATC 1440
                                                                  *           *

CitLGT-1   1441:CACAGAGTTAAGGAATTAGTGGAGAAGACGGCAACGGCAACTGCAAATGACAAGGTAGAATTGGTGGAGTCACGACGGACACGTGTACAG 1530
CitLGT-2   1441:CACAGAGTTAAGGAATTAGTGGAGAAGACGGCAACGGCAACTGCAAATGACAAGGTAGAATTGGTGGAGTCACGACGGACACGTGTACAG 1530
                                                                                      <----------
                                                                                      LGT-GR

CitLGT-1   1531:TATTGA                                                                            1536
CitLGT-2   1531:TATTGA                                                                            1536
```

Figure 2. (A) Comparison of nucleotide sequences between *CitLGT-1* and *CitLGT-2*. Asterisks indicate different nucleotides between *CitLGT-1* and *CitLGT-2*. Arrows indicate primer site used in this study. The recognition sequences of *Hinc*II are underlined. (B) Restriction map of *CitLGT*s digested with *Hinc*II. *CitLGT-1* has been registered in DDBJ (accession number AB033758).

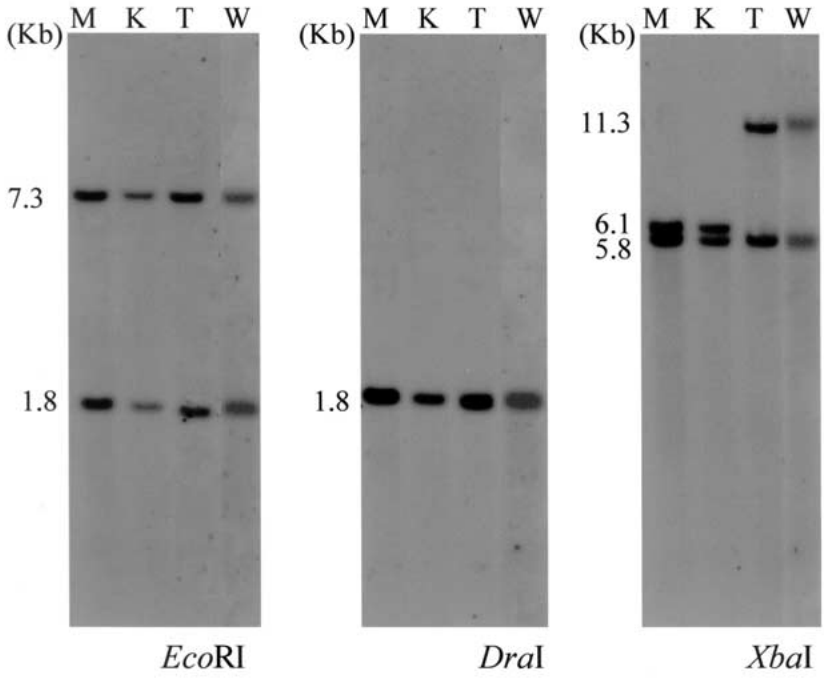

Figure 3. Southern blot analysis of the gene for *CitLGT* against the genomic DNA of Miyagawa-wase (M), Kiyomi tangor (K) (Miyagawa-wase × Trovita orange (T)), Trovita orange, and Washington navel orange (W). Total DNA (10 μg) was digested with *Eco*RI, *Dra*I or *Xba*I and separated on 1.0% agarose gel. It was blotted on nylon membrane (Hybond-NX). The blot was hybridized with Dig-11-dUTP labeled cDNA and washed twice with 0.1 × SSC and 0.1% SDS at 65 °C for 15 min and exposed to X-ray film (RX-U, Fuji Film).

darin. The sequence of an amplified genomic fragment of Satsuma mandarin revealed that two types of *CitLGT* were harbored in the genome of Satsuma mandarin. One was same as the previously isolated *CitLGT* cDNA sequence (Kita et al., 2000), and other differed by 15 bases from the previous *CitLGT* (Figure 2A). Other cultivars of navel orange and Satsuma mandarin showed the same respective restriction patterns as Washington navel and Miyagawa-wase (data not shown). These results indicate that navel orange and Satsuma mandarin possess one and two types of *CitLGT* genomic sequences, respectively, and this characteristic is held by each species. Navel orange had only the same sequences as *CitLGT*. Therefore, we designated the previously isolated *CitLGT* as *CitLGT-1* and the new sequence in Satsuma mandarin as *CitLGT-2*. That is, navel orange only possesses *CitLGT-1*, while Satsuma mandarin possesses both *CitLGT-1* and *CitLGT-2*. In *CitLGT-2*, since the 968th nucleotide of *CitLGT-1* substituted to G from A (Figure 2A), the 961 bp fragment of Satsuma mandarin newly appeared without a restriction site for *Hinc*II (Figures 1B and 2B). The predicted amino acid sequence of CitLGT-2 differs from that of CitLGT-1 by 12 amino acids as 3 of 15 nucleotide substitutions in CitLGT-2 do not affect the translation products. Although these substitutions were scattered throughout the coding regions (Figure 2A), the sequences of the two conserved domains (N-terminal transmembrane domain and UDP-glucose binding domain) are not changed, indicating that CitLGT-2 is expected to have UDP-limonid:glucosyltransferase activity.

To investigate whether *CitLGT-1* and *CitLGT-2* are derived from a single locus, the genome structure of the locus was analyzed. Southern blot hybridization showed one or two bands in the *Citrus* species examined (Figure 3), indicating that most likely there is no difference in the copy number of the limonoid glucosyltransferase gene in the genome. Furthermore, as shown in Figure 4, the progeny test of the PCR-RFLP pattern of partial *CitLGT* (1–1259 bp region) between Miyagawa-wase and Kiyomi tangor detected only three types with *CitLGT-1/CitLGT-1*, *CitLGT-1/CitLGT-2*, and *CitLGT-2/CitLGT-2*. Because Kiyomi tangor is a hybrid of Miyagawa-wase and Trovita orange (predicted to be homozygous for

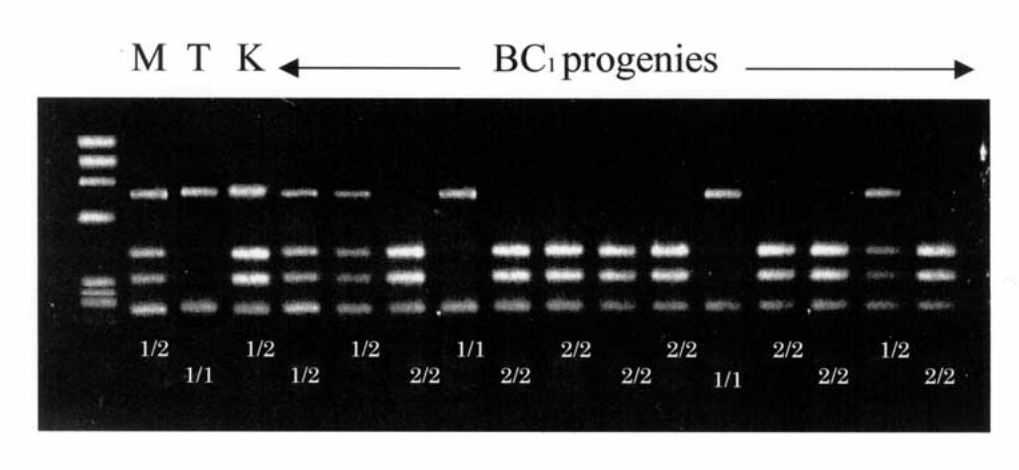

Figure 4. PCR-RFLP pattern of *CitLGT* partial region (1–1259 bp) in BC_1 populations between Miyagawa-wase (M) and Kiyomi tangor (K) (Miyagawa-wase × Trovita orange (T)). PCR products were digested by *Mbo*I and separated on 1.5% agarose gel (B). Numbers indicate the predicted allelic structure of *CitLGT*s (1/1: homozygous of *CitLGT-1*, 1/2: heterozygous of *CitLGT-1* and *CitLGT-2*, 2/2: homozygous of *CitLGT-2*). Marker: $\phi \times 174$/*Hae*III.

CitLGT-1), this result agrees with the previous report that *CitLGT* is a single locus gene (Kita et al., 2000) and fits the hypothetical segregation when Kiyomi tangor is heterozygous of *CitLGT-1* and *CitLGT-2*. Judging from the Southern blot hybridization (Figure 3) and the PCR-RFLP of the progenies (Figure 4), we conclude that *CitLGT-1* and *CitLGT-2* are alleles of a single gene. Navel orange is homozygous for *CitLGT-1*, and Satsuma mandarin is heterozygous for *CitLGT-1* and *CitLGT-2*.

The primers LGT-A1 and LGT-A2, which can be amplified to *CitLGT-1* and *CitLGT-2*, respectively, were synthesized based on three substituted nucleotides in the neighborhood of nucleotide 1289th (Figure 2A). PCR was performed using these selective primers against the first-strand cDNA from the juice sacs/segment epidermis of Satsuma mandarin and navel orange. As a result, in navel orange, the amplification of *CitLGT-1* was detected after 120 DAF; *CitLGT-1* was strongly amplified parallel to fruit maturation, but because navel orange kept only *CitLGT-1* in the genome, *CitLGT-2* was not (Figure 5). This result also shows that the selective primers, LGT-A1 and LGT-A2, are only three bases different each other and can alternatively recognize *CitLGT-1* and *CitLGT-2*, respectively.

Limonoid glucoside contents in the juice sacs/segment epidermis in Washington navel were low (under 50 ppm) until 120 DAF, when they began to increase rapidly and reached a maximum (428 ppm) at 180 DAF (Figure 5). On the other hand, in Satsuma mandarin, *CitLGT-2* was amplified strongly throughout all stages of development, while *CitLGT-1* was amplified beginning at 120 DAF (Figure 5). Limonoid glucosides in Miyagawa-wase accumulated even at the early stage of development and maintained high levels (over 200 ppm) throughout development (Figure 5). These results suggest that the presence of *CitLGT-2* is a prerequisite for the accumulation of non-bitter limonoid glucosides in the early- to mid-developmental stages of fruit.

Miyake et al. (1993) reported that the conversion of limonoid aglycones to limonoid glucosides takes place in the fruit at the late developmental stage and in the seed. In our study, the pattern of limonoid glucoside accumulation in Washington navel followed this report. However, in Miyagawa-wase, limonoid glucosides accumulated in fruit at all developmental stages. The different patterns between limonoid glucoside accumulation and *CitLGT*s expression observed in Satsuma mandarin and navel orange are due to the difference in the allelic structure of the *CitLGT*s.

In fruit trees, there have been limited investigations on the relationships between the allelic gene structures and the phenotypic fruit characteristics. Sunako et al. (1999) reported that allelism in the 1-aminocyclopropane-1-carboxylic acid synthase gene (ACS1) contributed to good, long-term storage properties of apple fruit. An allele in *Md-ACS1* generated by a retrotransposon-like SINE insertion in the promoter region (Sunako et al., 1999) was transcribed at a lower level than the original allele, resulting in low ethylene

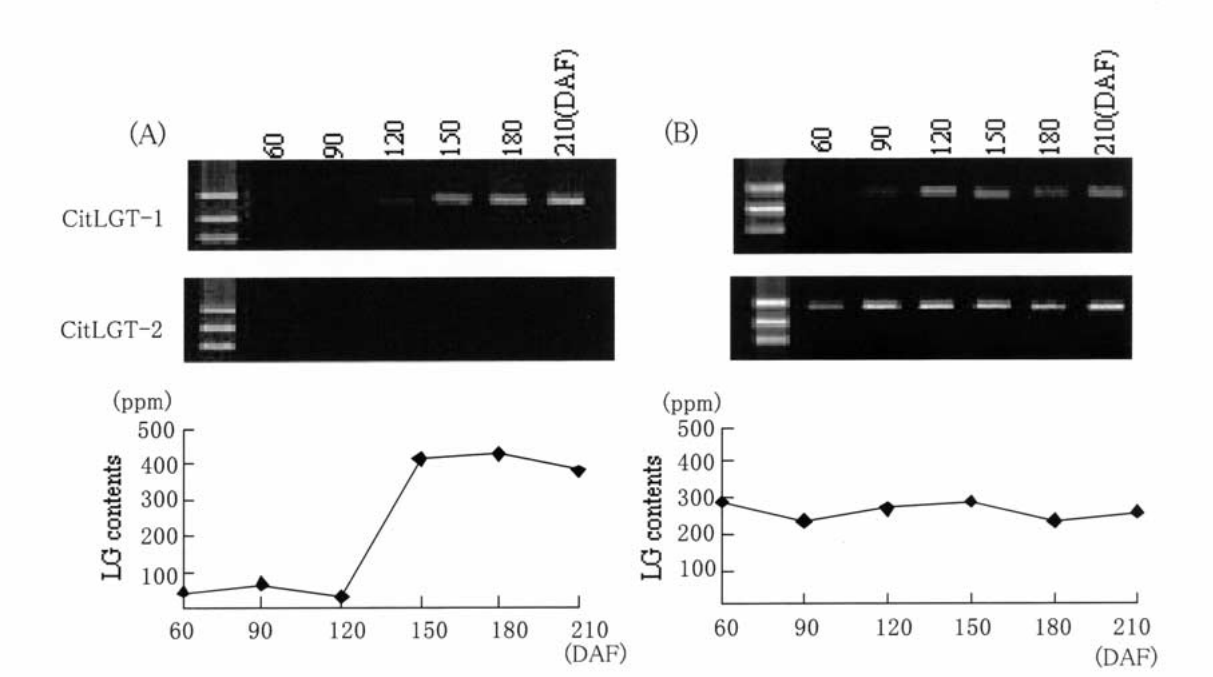

Figure 5. RT-PCR of *CitLGT*s and limonin glucoside contents in Washington navel (A) and Miyagawa-wase (B). PCR products were separated on 1.5% agarose gel. Limonin glucoside contents are indicated as the average of 2 measurements using HPLC. Marker: 3 fragments of $\phi \times 174$/*Hae*III (1357, 1078, and 872 bp fragments).

production and a longer shelf life of fruit. In general, the interaction between transposable elements and the regulatory sequences of genes can lead to alterations in the transcription (Weil & Wessler, 1990). Such a difference in the promoter region of the *CitLGT* gene may regulate the transcription levels of the alleles of the *Citrus* genome because there were many retrotransposable elements present (Asíns et al. 1999). A detailed analysis of the promoter structures of *CitLGT* alleles has yet to be done, but there was enough information on the coding regions of *CitLGT*s to develop molecular indicators for levels of limonoid glucoside accumulation in Satsuma mandarin and navel orange.

In summary, the present study shows that Satsuma mandarin (Miyagawa-wase) and navel orange (Washington navel) are discriminated by the specific primers for each *CitLGT*. These primers are useful for predicting limonoid glucoside levels that will accumulate in immature fruit. These molecular indicators will make it possible to characterize fruit traits at the seedling stage, resulting in the marker-assisted selection of hybrid progenies to predetermine whether they will accumulate non-bitter limonoid glucosides.

Acknowledgements

We thank Dr Berhow for his critical assistance in the reading and revision of this manuscript.

References

Asíns, M.J., A.J. Monforte, P.F. Mestre & E.A. Carbonell, 1999. *Citrus* and *Prunus copia*-like retrotransposon. Theor Appl Genet 99: 503–510.

Dellaporta, S.L., J. Wood & J.B. Hicks, 1983. A plant DNA minipreparation: version II. Plant Mol Biol Rep 1: 19–21.

Hasegawa, S., C.G. Suhayda, W.J. Hsu & G.H. Robertson, 1997. Purification of limonoid glucosyltransferase from navel orange albedo tissues. Phytochemistry 46: 33–37.

Hashinaga, F., H. Ejima, H. Nagahama & S. Itoo, 1977. Studies on the limonoids in Citrus fruit. Bull Fac Agric Kagoshima Univ 27: 171–179.

Ikoma, Y., M. Yano, K. Ogawa, T. Yoshioka, Z.C. Xu, S. Hisada, M. Omura & T. Moriguchi, 1996. Isolation and evaluation of RNA from polysaccharide-rich tissues in fruit for quality by cDNA library construction and RT-PCR. J Jpn Soc Hort Sci 64: 809–814.

Kita, M., Y. Hirata, T. Moriguchi, T. Endo-Inagaki, R. Matsumoto, S. Hasegawa, C.G. Suhayda & M. Omura, 2000. Molecular cloning and characterization of a novel gene encoding limonoid UDP-glucosyltransferase in *Citrus*. FEBS Lett 469: 173–178.

Lam, L.K.T. & S. Hasegawa, 1989. Inhibition of benzo[α]pyrene-induced forestomach neoplasia in mice by citrus limonids. Nutr Cancer 12(1): 43–47.

Lam, L.K.T., Y. Li & S. Hasegawa, 1989. Effects of citrus limonoids on glutathione S-transferase activity in mice. J Agric Food Chem 37: 878–880.

Lam, L.K.T., J. Zhang, S. Hasegawa & H.A.J. Schut, 1994. Inhibition of chemically induced carcinogenesis by citrus limonids. In: M. Huang, T. Osawa, C. Ho & R.T. Rosen (Eds.), Food Phytochemicals for Cancer Prevention I. ACS symposium series 546, pp. 209–219. American Chemical Society, Washington, D.C.

Mayer, V.P. & G.D. Beverly, 1968. Limonin monolactone, the nonbitter precursor responsible for delayed bitterness in certain citrus juices. J Food Sci 33: 488–492.

Mayer, V.P., S. Hasegawa & E. Here, 1969. Limonin D-ring lactone hydrolase. A new enzyme from Citrus seeds. Phytochemistry 8: 405–407.

Miller, E.G., A.P. Gonzales-Sanders, A.M. Couvillon, W.H. Binnie, S. Hasegawa & L.K.T. Lam, 1994. Citrus limonoids as inhibitors of oral carcinogenesis. Food Technol: 110–114.

Miyake, M., S. Ayano, Y. Ozaki, Z. Herman & S. Hasegawa, 1992. Limonoids in seeds of sour orange (*Citrus aurantium*). Nippon Nogeikagaku Kaishi 66: 31–34 (in Japanese).

Miyake, M. & S. Hasegawa, 1993. Biochemistry of citrus limonoids and their anticarcinogenic activity. The Food Industry 36(24): 26–34 (in Japanese).

Ozaki, Y., S. Ayano, N. Inaba, M. Miyake, M.A. Berhow & S. Hasegawa, 1995. Limonoid glucoside in fruit, juice and processing by-products of Satsuma mandarin (*Citrus unshiu* Marcov.). J Sci Food 60: 186–189.

Sunako, T., W. Sakuraba, M. Senda, S. Akada, R. Ushikawa, M. Niizeki & T. Harada, 1999. An allele of the ripening-specific 1-Aminocyclopropane-1-carboxylic acid synthase gene (*ACS1*) in apple fruit with a long storage life. Plant Physiol 119: 1297–1303.

Weil, C.F. & S.R. Wessler, 1990. The effects of plant retrotransposable elements insertion on transcription initiation and RNA processing. Annu Rev Plant Mol Biol 41: 527–552.